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14. ABSTRACT

We proposed to test a hypothesis that cell fusion between tumor cells and between tumor and normal cells contributes to metastasis. This contribution can be implemented by two mechanisms, by generating cells with diverse genetic and epigenetic properties, and by providing tumor cells with qualities of normal cells that are required to reside in normal tissues. This hypothesis might explain why cells tumor cells can grow at distant sites, why they express proteins that are normally expressed by cells of the metastasized tissue, and why only a minute fraction of cells released by the primary tumors form metastases. The funded research focuses on two specific aims, to determine the mechanism of gene transfer between prostate cancer cells (Aim 1); and to determine whether cell fusion affects metastatic properties of prostate cancer cells (Aim 2).

During the previous report period, we made an unexpected finding that a virus transfers genetic markers among the prostate cancer cells that we were using. Since then, we obtained a partial sequence of the viral genome, which is consistent with the possibility that this virus is related to XRMV, a virus isolated from prostate cancer biopsies. We will continue to characterize this potentially new virus. To accomplish Aim 2, we optimized production of cell hybrids and began determining their metastatic and tumorigenic properties.

15. SUBJECT TERMS
None listed.

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INTRODUCTION: The main goal of the research funded by this grant is to test the hypothesis that fusion among tumor cells or fusion of tumor to normal cells facilitates metastasis. The initial observation was the finding that human prostate cancer cells (PC3) that were transduced either with green fluorescent protein EGFP ("green" PC3 cells) or red fluorescent protein RFP ("red" PC3 cells) injected into mice produced tumors composed of cells that expressed both proteins ("yellow" cells). The "yellow" cells had enhanced metastatic potential, which suggested that the horizontal exchange of the genetic information affected cell malignancy. We proposed to identify the mechanism of genetic exchange (Aim 1), with the main hypothesis being that the gene exchange was mediated by cell fusion, and to test whether cell fusion caused by viruses can affect the ability of PC3 cells to metastasize (Aim 2).

KEY RESEARCH ACCOMPLISHMENTS:

AIM 1. The original title of this Aim was "To determine the mechanism of gene transfer between prostate cancer cells". Our initial hypothesis was that gene transfer in this experimental system is mediated by cell fusion. However, during the first year of this project we made several observations that were consistent with the possibility that genetic markers were transferred by a virus. We proposed two hypotheses regarding the origin of this virus. The first was that "green" and "red" PC-3 cells were infected by a xenotropic mouse virus, such as mouse leukemia virus (MLV) during the propagation of the cells in the animals. The second hypothesis was that the virus was present in the original PC-3 cells and was activated by experimental manipulations, was suggested by discovery of XRMV, a virus recovered from human prostate cancers, yet closely related to the xenotropic mouse viruses(Urisman et al., 2006). Testing our hypotheses required identifying the virus.

As we previously reported, we isolated the virus(es) from tissue culture medium conditioned by "green" PC-3 cells and identified by mass spectrometry some of the isolated polypeptides as fragments of Gag and Env of the mouse leukemia virus (MuLV). These results were consistent with either of our hypotheses but could not distinguish between them, as XRMV and MuLV are 95%-98% identical. Therefore, during the last year, we attempted to obtain the sequence of the viral genome.

Our initial attempts were unsuccessful, even though in the control experiments we could easily isolate and identify MuLV from mouse cells infected with this virus. However, recently, we found conditions that yielded three independent overlapping clones of the

viral DNA representing 1457 nucleotides of the gag gene, or about 20% of the genome size expected from an MuLV or a related virus. The obtained sequence was not fully identical to any sequence in the non-redundant NCBI nucleotide database (top hits are listed in Table 1), 98% identical to either MuLV or to XRMV isolates VP42 and VP35, 97% to isolate VP62, and 98% to the DG-75 Murine leukemia virus, which is constitutively released by a human B-lymphoblastoid cell line (Raisch et al., 2003). All detected mismatches were single nucleotide substitutions.

The obtained results were consistent with the possibility that the virus that we isolated was a new entity, which was intriguing, as it would include the possibility of human origin, but failed to indicate whether the virus originated from PC-3 cells or was contracted by the cells from the mouse. We are currently obtaining the remaining sequence of the viral genome. We will use the obtained information to answer the following basic questions. Was this virus, which we will call for convenience XRMV2, contracted from mice, present in the original PC3 cells, or introduced while creating the "green" and "red" PC-3 cells? Does XRMV2 transform normal human cells? Does XRMV2 affect cell proliferation or viability? Is XRMV2 present in other prostate cancer cell lines? If we find that XRMV2 is present in the original PC-3 cells or in another prostate cancer cell line, we will establish a collaboration to determine if this virus is also present in prostate cancer biopsies. We envision that answering these questions fully will likely require additional funding, which we hope to secure using preliminary results obtained by accomplishing this Aim.

AIM 2. To determine whether cell fusion affects metastatic properties of prostate cancer cells. The main goal of this aim was to test whether fusion of prostate cancer cells whether to themselves or to normal cells of the host affects the rate or tropism of metastasis. By design, research proposed in this Aim was independent from the results obtained in Aim 1. However, the unexpected finding that viruses, which we planned to use to fuse cells, could be involved in horizontal gene transfer, led us to look for another approach to cell fusion. During the first year of the project we developed a new approach for cell fusion that relied on the ability of VSVG, a viral fusogenic protein, to be reversibly activated in acidic medium. During the last year, we applied this approach to obtain clonogenic hybrids between PC-3-GLA (PC-3 cells transduced with a retroviral vector expressing Lamin A-EGFP, which is used as a fluorescent tracer) and PC-3-CIV (PC-3 cells transduced with a retroviral vector expressing mCherry as a fluorescent tracer and

VSVG). We recently injected these hybrids as well as the parental cell lines into the first series of nude mice and within three months we expect to learn whether the hybrids differ from the parental cells, or from the original PC-3 cells in their ability to form tumors or metastases. As was proposed in our application we are currently beginning to develop approaches for isolating hybrids between PC-3 cells and normal cells, such as macrophages to test whether fusion to normal cells of hematopoietic origin facilitates metastasis.

Our experience with optimizing production of clonogenic hybrids made us realize that we poorly understand how clonogenic survival of fused cells or cells that become tetraploid by other means is regulated. Considering that tetraploidy is considered an intermediate stage in carcinogenesis, we developed an experimental system to identify molecular

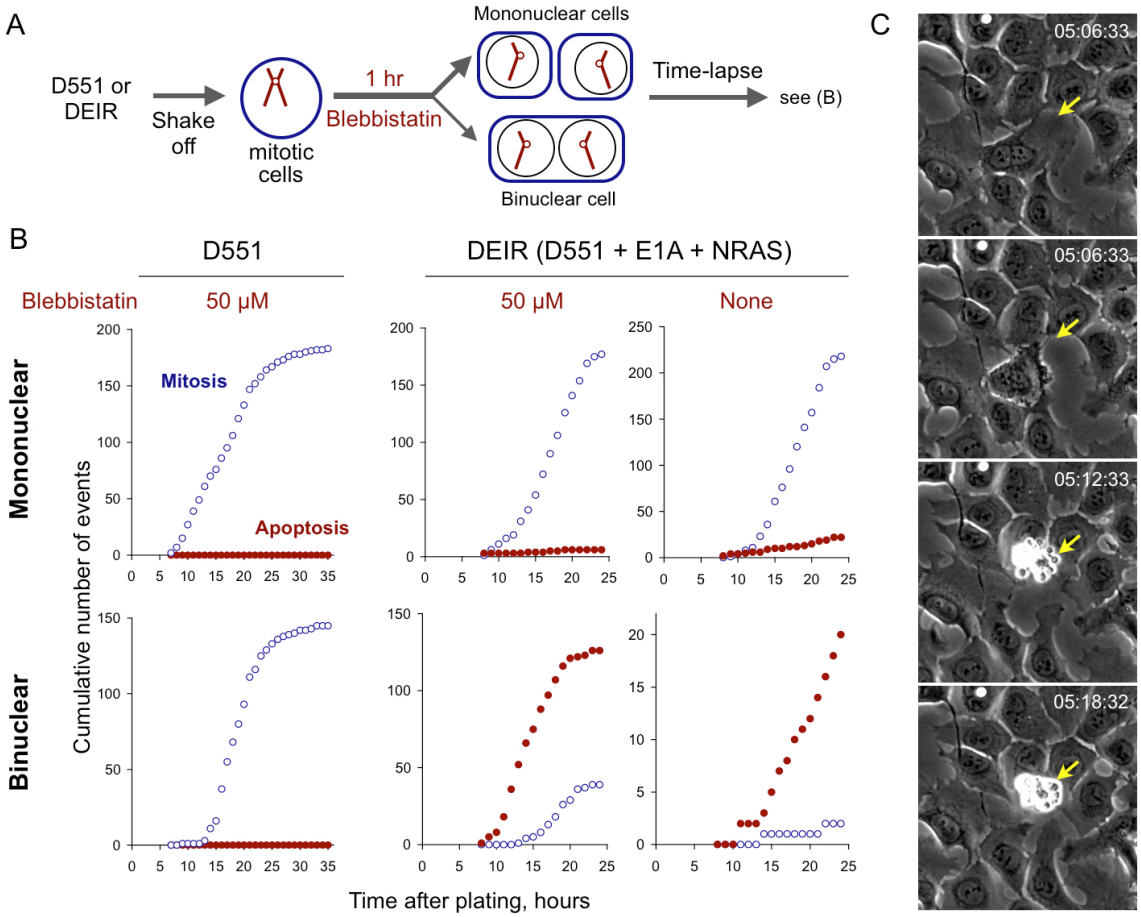

Figure 1. Preventing cytokinesis causes oncogene-dependent apoptosis. A. Experimental design. Mitotic cells were obtained by shake-off from asynchronous cell populations of normal human fibroblasts Detroit 551 (D551) or DEIR cells (D551 transduced with a retroviral vector expressing E1A and oncogenic NRAS). The mitotic cells were plated in medium containing either 50 μM blebbistatin or no drug. The cells were washed one hour later four times to remove the drug and monitored by time lapse microscopy for the incidence of mitosis and apoptosis, which were plotted in **(B)** as a cumulative number of events over the time elapsed after cell plating. An example of a typical even that was scored as apoptosis is presented in C. The time stamps in (C) indicate hours:minutes:seconds from the start of the experiment. The results in (B) are a summary of two independent experiments. DEIR cells were used within 10 passages following retroviral transduction.

pathways regulated by tetraploidy in premalignant cells (Figure 1).

In this experimental system, normal diploid human fibroblasts are co-transduced with adenoviral oncogene *E1A*, which deregulates cell cycle by disabling several key transcriptional regulators, including Rb, and an oncogenic mutant of *RAS,* which has multiple effects, including inhibiting apoptosis that occurs when 'normal' cells are forced to proliferate by the expression of E1A. The resulting cells have epithelioid morphology, express markers of epithelial cells, proliferate in soft agar and, if provided with an additional oncogene or are fused, make tumors in nude mice.

To cause tetraploidy we used an established approach (Fig. 1A) in which mitotic cells obtained from an asynchronous population were incubated with blebbistatin at a

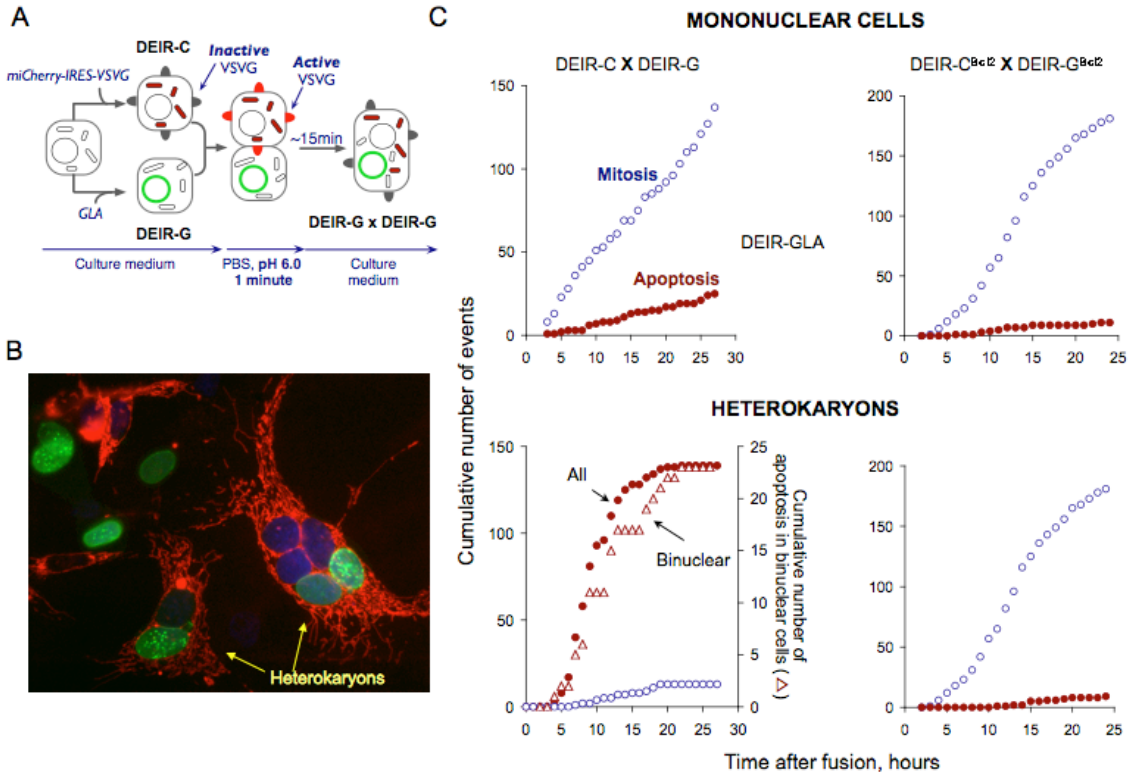

Figure 2. Cell fusion induces apoptosis. A. Cell fusion approach. One of the fusion partners is transduced with the gene encoding VSVG, the fusion protein of the vesicular stomatitis virus, which is inactive at normal pH of tissue culture medium, but can be ***reversibly*** activated by changing the pH to 6.0, thus initiating fusion of adjacent cells. The heterokaryons can be identified using fluorescent markers (B), such as mitochondria-targeted mCherry (red), and fusion of lamin A to EGFP (green), or by phase microscopy as cells with multiple nuclei. C. Cell fusion induces apoptosis. DEIR cells expressing one of the two fluorescent markers (left column) or, in addition, Bcl-2 (right column) were fused as described in (A). The heterokaryons and unfused (mononuclear) cells were then monitored by time-lapse microscopy to record the incidences of mitosis and apoptosis, which were plotted in C. The results are from one experiment of several experiments.

concentration low enough to make only some cells binuclear, while others were able to

complete cytokinesis, producing mononuclear cells. Thus, we compared the fate of cells that were treated identically in the same dish, but became either binuclear (tetraploid) or mononuclear (diploid). We used time-lapse microscopy to score mitosis, apoptosis, and cell cycle arrest. To avoid cytostatic effects of intense illumination required for fluorescence microscopy we relied solely on phase contrast illumination.

The majority of binuclear fibroblasts (Fig 1, D551) completed the first cell cycle and mitosis, consistently with the reports that tetraploidy does not cause cell cycle arrest. However, binuclear D551 transduced with *E1A* and *NRAS* (DEIR cells) died by apoptosis (Fig. 1B) as indicated by changes in morphology and the ability of the apoptosis inhibitor Bcl-2 to completely prevent cell death. Apoptosis was caused by cytokinesis failure and not by side effects of blebbistatin, because mononuclear cells remained viable (Fig 1B) and binuclear cells that were formed occasionally in the absence of blebbistatin (Fig. 1B, right column) also underwent apoptosis. Inhibiting cytokinesis by a distinct drug, an inhibitor of aurora kinase ZM447439, provided similar results. Overall, we concluded that failure to divide induced apoptosis.

We also found that cell fusion, which causes tetraploidy by a different mechanism, also causes apoptosis, which could be inhibited by expression of Bcl-2 (Fig. 2A,B). In a separate set of experiments we found that heterokaryons made from cells synchronized in the G1 phase also died, indicating that apoptosis was not caused by cell cycle asynchrony. Therefore, we concluded that events that increase ploidy cause apoptosis.

However, preventing apoptosis induced either by cell fusion or failed cytokinesis failed to make the resulting cells clonogenic. Therefore, apoptosis is only one of the mechanisms that prevent clonogenic expansion of tetraploid cells.

Some tetraploid cells remained clonogenic if the parental cells expressed a dominant negative mutant of the tumor suppressor p53 (p53R175H), which also prevented apoptosis. Because preventing apoptosis failed to make cells clonogenic, while expression of p53DN did, we concluded that p53DN enabled clonogenicity of tetraploid cells through a mechanism unrelated to apoptosis.

Overall, this “side-project” has allowed us to better understand better how to produce clonogenic hybrids and also has provided an experimental system to dissect regulation of viability in tetraploid premalignant cells. We used preliminary results obtained with this system to begin a collaboration with Drs. Andrew Koff and Hakim Djaballah (Memorial

Sloan-Kettering Cancer Center) to identify molecular pathways and small molecules that regulate clonogenic survival of tetraploid cells. We applied for funding to support this collaborative effort.

REPORTABLE OUTCOMES: We developed an experimental system to identify the pathways controlling clonogenic expansion of tetraploid premalignant cells and generated a series of plasmids and cell lines that will be made available to the scientific community once our results are reported.

CONCLUSIONS: Overall, by accomplishing Aim 1 we unexpectedly entered an area of cancer biology – a relationship between viral infections and cancer – from a perspective that may be unrelated to cell fusion but might provide new insights into an intriguing link between human prostate cancer and viruses closely related to mouse leukemia virus. Identifying the genome of the XRMV2 will help us to develop tools for determining the origin of the virus, its properties, and its relationship to prostate cancer. We have continued to work on Aim 2, an effort that will be helped by a better understanding of the consequences of cell fusion for clonogenic survival.

REFERENCES:

Raisch, K.P., M. Pizzato, H.Y. Sun, Y. Takeuchi, L.W. Cashdollar, and S.E. Grossberg. 2003. Molecular cloning, complete sequence, and biological characterization of a xenotropic murine leukemia virus constitutively released from the human B-lymphoblastoid cell line DG-75. *Virology*. 308:83-91.

Urisman, A., R.J. Molinaro, N. Fischer, S.J. Plummer, G. Casey, E.A. Klein, K. Malathi, C. Magi-Galluzzi, R.R. Tubbs, D. Ganem, R.H. Silverman, and J.L. DeRisi. 2006. Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. *PLoS Pathog.* 2:0211-0225.

BLAST Basic Local Alignment Search Tool

Table 1

-

Edit and Resubmit Save Search Strategies Formatting options Download

Nucleotide Sequence (1457 letters)

Results for: lcl|13191 None(1457bp)

Your BLAST job specified more than one input sequence. This box lets you choose which input sequence to show BLAST results for.

Query ID
lcl|13191
Description
None
Molecule type
nucleic acid
Query Length
1457

Database Name
nr
Description
All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
Program
BLASTN 2.2.19+ Citation

Reference
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.
Other reports: Search Summary [Taxonomy reports] [Distance tree of results]

Search Parameters

Program	blastn
Word size	28
Expect value	10
Hitlist size	100
Match/Mismatch scores	1,-2
Gapcosts	0,0
Low Complexity Filter	Yes
Filter string	L;m;
Genetic Code	1

Database

Posted date	Mar 4, 2009 5:51 PM
Number of letters	26,448,767,150
Number of sequences	8,301,951
Entrez query	none

Karlin-Altschul statistics

Params	Ungapped	Gapped
Lambda	1.33271	1.28
K	0.620991	0.46
H	1.12409	0.85

Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

Sequences producing significant alignments:
(Click headers to sort columns)

AC127575.4	Mus musculus BAC clone RP24-240L12 from chromosome 8, complete sequence	2608	2608	100%	0.0	98%	
AC121813.3	Mus musculus BAC clone RP23-457E5 from 9, complete sequence	2601	2601	100%	0.0	98%	
AC163703.4	Mus musculus BAC clone RP23-359B23 from chromosome 8, complete sequence	2597	2597	100%	0.0	98%	
AC167466.6	Mus musculus chromosome 7, clone RP24-220N8, complete sequence	2597	2597	100%	0.0	98%	
AC102432.9	Mus musculus chromosome 19, clone RP24-114A21, complete sequence	2597	2597	100%	0.0	98%	
AC161413.5	Mus musculus chromosome 19, clone RP23-106D17, complete sequence	2597	2597	100%	0.0	98%	
AC159275.6	Mus musculus BAC clone RP23-367C13 from chromosome 8, complete sequence	2597	2597	100%	0.0	98%	
AL627077.14	Mouse DNA sequence from clone RP23-384D6 on chromosome 4 Contains the 5' end of the gene for a novel KRAB box containing protein, a pseudogene similar to part of tousled-like kinase 2 (Arabidopsis) Tlk2, a protein phosphatase 2, regulatory subunit B (B56), alpha isoform (Ppp2r5a) pseudogene, a high mobility group box 2 (Hmgb2) pseudogene, a novel gene, the gene for a novel KRAB box containing protein, a ribosomal protein, large, P1 (Rplp1) pseudogene, a carnitine deficiency-associated gene expressed in ventricle 3 (Cdv3) pseudogene, the 3' end of the gene for a novel KRAB box containing protein and four CpG islands, complete sequence	2595	2595	100%	0.0	98%	
AF221065.1	DG-75 Murine leukemia virus, complete genome	2591	2591	100%	0.0	98%	
AC164433.4	Mus musculus BAC clone RP23-357G5 from chromosome 14, complete sequence	2575	2575	100%	0.0	98%	
CT030655.7	Mouse DNA sequence from clone RP23-259C9 on chromosome 13, complete sequence	2569	2569	100%	0.0	98%	
AL929426.8	Mouse DNA sequence from clone RP23-471H23 on chromosome 11 Contains the 3' end of the Scpep1 gene for serine carboxypeptidase 1, complete sequence	2564	2564	100%	0.0	98%	
AY999005.1	Mus musculus castaneus endogenous virus Rmcf2, complete sequence	2553	2553	100%	0.0	98%	G
EF185282.1	Xenotropic MuLV-related virus VP62, complete genome	2547	2547	100%	0.0	98%	G
AC114666.31	Mus musculus chromosome 5, clone RP24-175N6, complete sequence	2547	2547	100%	0.0	98%	
AC113104.15	Mus musculus chromosome 18, clone RP23-321L15, complete sequence	2547	2547	100%	0.0	98%	
AC103621.6	Mus musculus chromosome 5, clone RP24-230J14, complete sequence	2547	2547	100%	0.0	98%	

AC158975.9	Mus musculus chromosome 18, clone RP24-192J22, complete sequence	2547	2547	100%	0.0	98%
DQ241302.1	Xenotropic MuLV-related virus VP42, complete genome	2547	2547	100%	0.0	98%
AL844206.20	Mouse DNA sequence from clone RP23-84A23 on chromosome 4 Contains the Scp2 gene for sterol carrier protein 2, liver, the 5' end of the Echdc2 gene for enoyl Coenzyme A hydratase domain containing 2 and a CpG island, complete sequence	2547	2547	100%	0.0	98%
AL845268.2	Mouse DNA sequence from clone RP23-69A6 on chromosome X Contains a ribosomal protein L18 (Rpl18) pseudogene, a transcription elongation factor B (SIII), polypeptide 1 (Tceb1) pseudogene and one CpG island, complete sequence	2547	2547	100%	0.0	98%
AC164119.4	Mus musculus BAC clone RP24-288I15 from chromosome 5, complete sequence	2542	2542	100%	0.0	98%
AC093473.6	Mus musculus chromosome 5, clone RP23-54H12, complete sequence	2542	2542	100%	0.0	98%
AC127266.4	Mus musculus BAC clone RP24-347B12 from chromosome 5, complete sequence	2542	2542	100%	0.0	98%
AC131728.4	Mus musculus BAC clone RP23-122G5 from chromosome 5, complete sequence	2542	2542	100%	0.0	98%
AC121777.3	Mus musculus BAC clone RP23-359A23 from chromosome 16, complete sequence	2542	2542	100%	0.0	98%
DQ241301.1	Xenotropic MuLV-related virus VP35, complete genome	2542	2542	100%	0.0	98%
AC153954.3	Mus musculus 10 BAC RP24-236E2 (Roswell Park Cancer Institute (C57BL/6J Male) Mouse BAC Library) complete sequence	2542	2542	100%	0.0	98%
AC154849.2	Mus musculus BAC clone RP23-35P8 from chromosome 13, complete sequence	2542	2542	100%	0.0	98%
AC123679.16	Mus musculus chromosome 5, clone RP23-280N22, complete sequence	2542	2542	100%	0.0	98%
AC127583.4	Mus musculus BAC clone RP24-121M11 from 13, complete sequence	2542	2542	100%	0.0	98%
CR954969.10	Mouse DNA sequence from clone RP23-332J14 on chromosome X Contains two Xlr-related, meiosis regulated (Xmr) family pseudogenes, complete sequence	2542	2542	100%	0.0	98%
AL844871.11	Mouse DNA sequence from clone RP23-271B15 on chromosome 2 Contains the Nr4a2 gene for nuclear receptor subfamily 4 group A member 2, two novel genes, a novel gene (4930555B11Rik), an ATP synthase H+ transporting mitochondrial F1F0complex subunit e (Atp5k) pseudogene, the 5' end of the Gpd2 gene for glycerol phosphate dehydrogenase 2 mitochondrial, a cytoskeleton associated protein 2 (Ckap2) pseudogene and seven CpG islands, complete sequence	2542	2542	100%	0.0	98%
AC139295.5	Mus musculus BAC clone RP24-131B6 from chromosome 1, complete sequence	2542	2542	100%	0.0	98%
CU407331.10	Mouse DNA sequence from clone CH29-	2536	2536	100%	0.0	98%

	519H15 on chromosome 11 Contains the 3' end of the Dgke gene for diacylglycerol kinase epsilon, the 5' end of the Scpep1 gene for serine carboxypeptidase 1, the Trim25 gene for tripartite motif protein 25, a transcription elongation factor B (SIII) polypeptide 2 (18kD, elongin B) Tceb2 pseudogene, the Coil gene for coilin, the 3' end of a novel (4930405P13Rik), two novel genes and three CpG islands, complete sequence					
AC167978.4	Mus musculus BAC clone RP23-72M20 from chromosome 7, complete sequence	2536	2536	100%	0.0	98%
AC112789.4	Mus musculus BAC clone RP23-151J21 from chromosome 7, complete sequence	2536	2536	100%	0.0	98%
AC153360.6	Mus musculus BAC clone RP23-321I6 from chromosome 10, complete sequence	2536	2536	100%	0.0	98%
AC164640.4	Mus musculus BAC clone RP23-228M12 from chromosome 7, complete sequence	2536	2536	100%	0.0	98%
AC122256.3	Mus musculus BAC clone RP23-178H7 from 7, complete sequence	2536	2536	100%	0.0	98%
AC154449.2	Mus musculus BAC clone RP24-168F5 from chromosome 16, complete sequence	2536	2536	100%	0.0	98%
AL589879.21	Mouse DNA sequence from clone RP23-38E20 on chromosome 13 Contains the genes for H2b histone family member A, two H2a, two H4 and an H2b histone family member, a serine/threonine protein kinase pseudogene, a novel pseudogene, a novel gene (4930500C15Rik), the gene for a novel KRAB box and C2H2 Zinc Finger containing protein, a ribosomal protein L21 (RPL21 pseudogene, a TU mitochondrial translation elongation factor (tufa pseudogene, the 5' end of the gene for a novel protein similar to nuclear envelope pore membrane protein POM121 and four CpG islands, complete sequence	2536	2536	100%	0.0	98%
AC165340.3	Mus musculus BAC clone RP24-63G4 from chromosome 7, complete sequence	2536	2536	100%	0.0	98%
AL645571.18	Mouse DNA sequence from clone RP23-39H1 on chromosome 11 Contains a general transcription factor II A, 1 (Gtf2a1) pseudogene, the 3' end of the Pkd1l1 gene for polycystic kidney disease 1 like 1 and a heterogeneous nuclear ribonucleoprotein A3 (Hnrpa3) pseudogene, complete sequence	2534	2534	100%	0.0	98%
AC163617.5	Mus musculus BAC clone RP23-115O21 from chromosome 8, complete sequence	2531	2531	100%	0.0	98%
AC123075.5	Mus musculus BAC clone RP23-181C16 from chromosome 3, complete sequence	2531	2531	100%	0.0	98%
AC114603.15	Mus musculus chromosome 1, clone RP23-355E19, complete sequence	2531	2531	100%	0.0	98%
AL731663.12	Mouse DNA sequence from clone RP23-360J20 on chromosome 4 Contains a tousled-like kinase 2 (Arabidopsis) (Tlk2) pseudogene, two novel genes, twp carnitine deficiency-associated gene	2531	2531	100%	0.0	98%